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Original Article

Molecular and Bioassay Examination of *Neospora Caninum* Infection in Bovine Aborted Fetuses in Khorasan Razavi Province, Iran

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ABSTRACT

Neospora caninum plays a significant role in causing abortion and reproductive failure in dairy cattle. The majority of neosporosis-related abortions occur during the 5–6 months of gestation. Outcomes can include fetal death within the uterus, resorption, mummification, autolysis, stillbirth, birth with clinical symptoms, or birth of clinically healthy but chronically infected ones. The objective of this study was to identify *N. caninum* infection in aborted bovine fetuses through molecular analysis and mouse bioassay testing. From 2019 to 2022, a total of 121 bovine aborted fetuses were collected from dairy farms in Khorasan Razavi province. Fetal brain samples were screened for detection of the parasite DNA using polymerase chain reaction assay (PCR). Additionally, a portion of PCR-positive brain tissue was homogenized and inoculated into the peritoneum of five BALB/c mice. All mice were sacrificed six weeks post -infection and examined using serology, microscopy and PCR methods. If the mice's brain samples tested PCR -positive, the mouse bioassay test was repeated two times. The *N. caninum* DNA was detected in 19.8% of brain samples in bovine aborted fetuses. Among PCR-positive brain samples, only ten samples were suitable for mouse bioassay examination. All inoculated mice remained seronegative and showed no clinical signs after three rounds of bioassays, although PCR testing of the brain samples of three mice groups were PCR-positive after repeated bioassays. The PCR results showed a moderate frequency of *N.caninum* infection in aborted bovine fetuses. Furthermore, the isolates obtained in this study demonstrated low pathogenicity in BALB/c mice, suggesting that they belong to an avirulent strain.

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1. Introduction

Neospora caninum is recognized as the primary cause of abortion in cattle worldwide (1). There are two main ways of transmission for *N. caninum* in cattle. The main method of infection, often referred to as vertical, congenital, or endogenous transmission, occurs when bardyzoites of cysts in the dam's tissues become active and transform into tachyzoites, which then cross through the placenta and infect the fetus. The secondary method, known as horizontal or post-natal transmission, happens when pregnant dairy cattle ingest sporulated *N. caninum* oocysts, allowing the sporozoites to transform into tachyzoites that likely disseminate through the circulation in cells of the mononuclear phagocytic system and potentially infect the fetus through transplacental transmission (4). Endogenous (vertical) transmission could occur in up to approximately 95% of infected dairy cattle (1, 7).

The high seroprevalence of *Neospora* infection has been reported in dairy cattle in Iran (8-11). Accordingly, studies have shown that *N. caninum* infection also contributes to abortion in dairy cattle (14, 15). Although there is a high prevalence of *N. caninum* infection in dairy cattle in Iran, only one *N. caninum* isolate has been recovered from an aborted bovine fetus (17). The present study aimed to detect the frequency of *N.caninum* infection in aborted bovine fetuses and evaluate the pathogenicity of *N. caninum* in BALB/c mice.

2. Materials and Methods

The study was conducted in Khorasan Razavi province, northern Iran, covering an area of over 127,000 km2 and located at coordinates 33°30′-37°41′ N; 56°19′-61°18′ E. This region experiences a semi-arid climate with a temperature zone characterized by cold winters and moderate summers There are approximately 25000 cattle distributed across 110 dairy farms in this region, with herd sizes ranging from 30 to 2000 cattle varying between farms. The predominant cattle breed in the area is Holstein-Friesian.

2.1. Sample collection

Between 2022 to 2023, a total of 121 aborted bovine fetuses were collected from different areas of the province. Thefetuses were necropsied, and brain tissue were collected for molecular and bioassay examination.

2.2. DNA Extraction and PCR

Samples were used to extract genomic DNA with the MBST Genomic DNA kit (Molecular and Biological Transmission Systems, Tehran, Iran) following the manufacturer's instructions. Then, we conducted a PCR assay to identify the *N.caninum* gene, following the previously described method by Müller et al 2002 (21). The simple PCR reaction was performed in a 25µl mixture containing 2µl of total DNA, 10µl of commercial premix master mix (Parstous Mashhad), 1µl of each primer, and 11µl of nuclease-free water in a thermocycler. The cycling process began with an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 63°C for 1 minute, and 74°C for 3.5 minutes, with a final extension step at 74°C for 10 minutes.

The Oligonucleotide primers used were NP21plus (5'CCCAGTGCGTCCAATCCTGTAAC3') and Np6plus (5'CTCGCCAGTCAACCTACGTCTTCT3').

2.3. Bioassay in Mice

Six to eight-week-old female BALB/c mice were obtained from the Razi Vaccine and Serum Research Institute (Mashhad Branch). The mice were housed in groups of five in plastic, cages with ad libitum access to rodent feed and water, under standard laboratory conditions. A total of 100 grams of brain tissue from PCR-positive aborted fetuses were homogenized in 500 ml of 0.85% NaCl solution (saline) containing antibiotics (100 IU/ml penicillin and 745 IU/ml streptomycin) and then homogenized using an electrical mixer. The mixture was subsequently filtered through two layers of gauze. After an incubation period of three hours at room temperature, the samples were then centrifuged at 1500 g for five minutes, and 5 ml of the homogenate deposit was administered intraperitoneally to 5 BALB/c mice (1 ml per mouse).

The mice were observed daily for any clinical signs indicating neosporosis. Blood samples were collected from the mice's tails six weeks after inoculation and their serum samples were analyzed using an Elisa kit (ID screen® N. caninum indirect Multi-species, ID. vet, Montpellier, France) to detect *Neospora* antibodies. All inoculated mice were euthanized 42 days post- infection using chloroform inhalation. Brain impression smears were prepared and examined under a microscope to detect cysts.

Additionally, a portion of the brain tissue was tested for *N.caninum* DNA using PCR. The PCR-positive brain samples from each group of mice were combined and then inoculated intraperitoneally into five BALB/C mice. These inoculated mice were monitored daily and euthanized seven weeks after inoculation.

During the necropsy, blood and brain samples were collected and analyzed using the serology and PCR methods as mentioned earlier. If the mice's brain samples tested positive via PCR, the mice bioassay was repeated to once again identify a viable cyst.

3. Results

In this study, *N.caninum* DNA was detected in 19.8% (24/121) of brain samples from bovine aborted fetuses (Figure 1). Among 24 PCR-positive brain samples, only 10 were suitable for mouse bioassay examination.

In the first bioassay round, all inoculated mice in ten groups were normal without clinical signs and the serology and microscopy results were also negative after 42 days post-infection. However, *N.caninum* -DNA was detected in five brain samples from two mice groups by PCR. Similar results were obtained after two rounds of mice bioassay examination using BALB/C mice inoculated with PCR-positive brain tissues from infected groups. (Table 1).

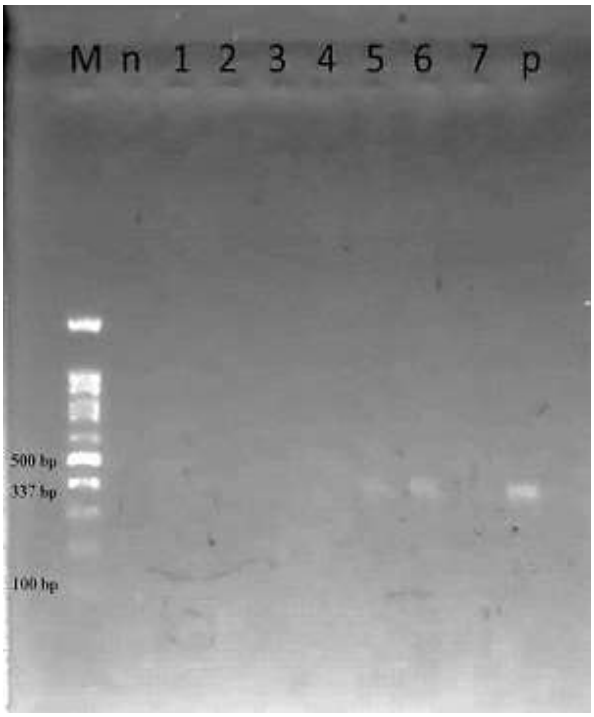

Figure 1. PCR amplification products of *N. caninum* in brain samples Lanes: M: molecular weight marker (between 1000 and 100bp); p: positive control (*Neospora* tachyzoites; 337 bp); n: negative control; 5, 6: positive samples.

Table1. The results of first, second and third round of mouse bioassay examination on PCR -positive bovine and mice brain samples.

Results	First round			Second round			Third round		
Group (5 mice)	bovine aborted fetuses (homogenized brain)			Mice			Mice		
	ELISA	Microscopy	PCR	ELISA	Microscopy	PCR	ELISA	Microscopy	PCR
1	N	N	N	nd	nd	nd	nd	nd	nd
2	N	N	N	nd	nd	nd	nd	nd	nd
3	N	N	P	N	N	P	N	N	P
4	N	N	P	N	N	N	nd	nd	nd
5	N	N	N	nd	nd	nd	nd	nd	nd
6	N	N	N	nd	nd	nd	nd	nd	nd
7	N	N	P	N	N	P	N	N	P
8	N	N	N	nd	nd	nd	nd	nd	nd
9	N	N	P	N	N	N	nd	nd	nd
10	N	N	P	N	N	P	N	N	P

P=Positive, N=Negative, nd= not done

4. Discussion

In this study, N. caninum DNA was identified in 19.8% (24 out of 121) of brain samples collected from aborted bovine fetuses using the PCR method. The reported prevalence of N. caninum infection in aborted bovine fetuses across various provinces in Iran range from 12% to 67%, as determined by PCR techniques (Table 2).

To date, virulent to avirulent strains of *N. caninum* have been reported by mice bioassay method (24, 27).

The BALB/C models have been used to assess the pathogenicity of *N. caninum* isolates from bovine aborted fetuses. Some isolates demonstrated low virulence, with no clinical symptoms or detectable *N.caninum* cysts in the mice brains (28-31).

Table 2. List of different studies frequency of *N. caninum* infection in brain tissue of aborted bovine fetuses in different areas of Iran.

province	Study Year	Method	Number examined	Number infected	Frequency	References
Khorasan Razavi	2007	PCR	6	4	67%	(2)
Khorasan Razavi	2010	PCR	151	18	12%	(3)
Khorasan Razavi	2013	PCR	200	23	12%	(5)
East Azerbijan	2013	PCR	14	6	43%	(6)
Charhar mahal & Bakhtiiari	2013	PCR	100	11	11%	(12)
Tehran	2014	PCR	16	12	75%	(13)
Qazvin	2014	PCR	128	39	31%	(16)
East Azerbaijan	2018	PCR	82	34	41%	(18)
Markazi	2018	PCR	38	10	26%	(19)
Mazandaran	2019	PCR	9	2	22%	(20)
Mazandaran	2021	PCR	78	16	20.5%	(22)

A meta-analysis indicated that the prevalence of *N. caninum* in aborted fetuses was higher in studies with fewer than 50 samples compared to those with more than 50 samples (23). The author concluded that the pooled estimate for studies with a sample size of 50 or more could provide a more accurate, conservative, and reliable representation of the overall infection rates in aborted bovine fetuses in Iran (23).

The brain tissue of bovine aborted fetuses is the primary source of *N.caninum* isolation (24), but some studies have shown that most *N.caninum* cysts in brain tissue were probably non-viable due to autolysis effect (25, 26). In this study, many positive brain samples were autolyzed after abortion, and only ten PCR-positive brain samples were suitable for bioassay examination. All inoculated mice in ten bioassay groups showed no clinical signs and no detectable *Neospora* cysts in their brain tissue. However, *N.caninum* DNA was detected in the brain samples of five mice in three bioassay groups. Similar results were obtained after conducting two additional rounds of mouse bioassays with PCR-positive brain samples. These findings suggest that these *N.caninum* isolates from aborted bovine fetuses were avirulent strains in BALB/C mice.

The PCR results confirmed the presence of N.caninum infection in the brain samples of aborted bovine fetuses and in two groups of inoculated mice. However, it remains unclear why no antibodies against N. caninum were found in the inoculated mice after three rounds of bioassays. Some studies also reported seronegative results for *Toxoplasma gondii* or *N.caninum* infection in different laboratory animals inoculated with infected mouse or rat brain tissue (32, 33).

It has been suggested that the absence of clinical signs and detectable antibodies against to *T.gondii* or *N.caninum* infection may result from rapid death of parasites in mouse neural tissue of mice, but had DNA intact present. The duration between brain tissue collection and analysis might have been too lengthy to support the viable tissue cysts in the brain tissue (33). In summary, this research demonstrated the presence of N.caninum infection in brains of aborted bovine fetuses in the Mashhad region. The result strongly suggests a high frequency of endogenous transmission among dairy cattle in Iran. In addition, bioassay results provide further evidence that *N.caninum* isolates might be an avirulent strain and need more investigation.

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Authors' Contribution

Study concept and design: G.R.R.
Acquisition of data: G.R.R.
Analysis and interpretation of data: A.K., M.S.,
Drafting of the manuscript: G.R.R.,
Critical revision of the manuscript for important intellectual content: G.R.R.,A.K., M.S.,
Statistical analysis: G.R.R.,A.K., M.S.,
Administrative, technical, and material support: G.R.R.
Study supervision: G.R.R.,A.K., M.S.,

Ethics

The mice were housed and maintained according to the guidelines of the animal care facility at Ferdowsi University of Mashhad. All animal experiments were performed in strict accordance with the protocols approved by the Animal Ethics Committee of our faculty (IR.UM.REC.1399.063).

Conflict of Interest

The authors declare no conflict of interest.

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Data Availability

The datasets generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

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